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# SAPONIN ADJUVANT ENHANCEMENT OF ANTIGEN-SPECIFIC IMMUNE RESPONSES TO AN EXPERIMENTAL HIV-1 VACCINE

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The adjuvant activity of a single highly purified saponin from the soap bark tree *Quillaja saponaria* was evaluated by using it as a component in an experimental vaccine containing rHIV-1 envelope protein (HIV-1 160D) adsorbed to alum. BALB/c mice immunized with experimental vaccine formulations containing the saponin adjuvant QS-21 produced significantly higher titers of antibodies than mice vaccinated with only the alum-adsorbed HIV-1 160D. Potent anamnestic antibody responses to HIV-1 viral proteins were also induced. Ag-specific proliferative responses to recombinant proteins and to three variants of HIV-1 were significantly increased using QS-21 as an adjuvant. Alum-adsorbed HIV-1 160D failed to induce measurable proliferative responses to inactivated HIV-1 viruses, but group-specific proliferative responses were raised when the QS-21 adjuvant was used in the vaccine formulation. MHC class I restricted CTL specific for the immunodominant V-3 loop were induced but only when the QS-21 adjuvant was included in the vaccine formulation. The production of serine esterase by Ag-activated splenic mononuclear cells, indicating the maturation of precursor CTL, was used as a secondary measure of CTL activity, and this response was also increased. The specificity of antibody responses was not significantly broadened using QS-21; the adjuvant increased the immune recognition of epitopes throughout the HIV-1 glycoprotein 160. However, the specificity of the proliferation and serine esterase responses was broadened, suggesting that the QS-21 augmented cell-mediated immune responses specific for epitopes outside of the V-3 loop. Additionally, the QS-21 adjuvant appeared to induce recognition of weakly immunogenic epitopes that were not recognized using only alum-adsorbed HIV-1 160D. The ability of QS-21 to augment both antibody and cell-mediated immune responses suggests that this adjuvant could be a valuable component in subunit vaccines.

responses after vaccination is critical if long lived protection against viral pathogens is to be achieved (1-4). Properties of this immune response must include the production of Ag-specific Th cells and CTL. Several experimental subunit vaccines have been developed for HIV-1 and tested using animal models. These subunit vaccines have been based on one or more HIV-1 structural proteins with the primary attention directed at the envelope glycoproteins, gp120<sup>2</sup> and rgp41, or the precursor protein, gp160. Vaccine formulations consisting of purified viral gp120, rgp120, or gp160 and synthetic peptides containing selected B and T lymphocyte epitopes have been shown to be immunogenic (5-13). Unfortunately, vaccines based on soluble proteins are not likely to induce the appropriate cell-mediated immune responses. The most significant limitation observed for experimental HIV-1 vaccines is that MHC class II Ag-restricted CTL were produced instead of MHC class I Ag-restricted CTL (14).

This limitation has stimulated research with recombinant viral proteins or synthetic peptides used in conjunction with adjuvants or carrier systems to augment immunogenicity. Several different adjuvants have been used as components in HIV-1 experimental vaccines including aluminum hydroxide (alum) (7, 11), IFA (11), poly (A,U), muramyl dipeptide (11), and bacterial lipopeptides (15). One promising source of adjuvants is the saponins from the soapbark tree, *Quillaja saponaria*. The saponins have been used with success in the commercially available form of Superfos Guil A in several experimental animal vaccines (16) and as the adjuvant active component in the carrier or Ag presentation system termed ISCOM (17). rHIV-1 gp160 incorporated into ISCOM has been used to induce MHC class I Ag-restricted CTL in mice (18).

Saponin-based adjuvants typically consist of a complex mixture of closely related triterpene glycosides, partially purified by extraction with aqueous solvents as mixed micelles. We have developed chromatography techniques using organic solvents to purify these mixtures of saponins into more than 28 fractions and identified in four different major fractions with adjuvant activity (19). For our experimental HIV-1 vaccine program we have used a single purified saponin, termed QS-21, in conjunction with a truncated and denatured rHIV-1 gp160. This ex-

<sup>2</sup> Abbreviations used in this paper: gp120, external envelope glycoprotein from HIV-1; gp41, transmembrane envelope glycoprotein of HIV-1; gp160 precursor form of envelope protein of HIV-1; V-3 loop, third variable region of the HIV-1 gp120; QS-21, adjuvant active reverse phase HPLC fraction 21 from *Q. saponaria* bark extract; IIIB, MN, and CC, variants of HIV-1; ISCOM, immunostimulating complex(es).

## The Induction of Ag-specific cell-mediated immune

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perimental vaccine formulation was tested in BALB/c mice to determine if a single saponin fraction could augment HIV-1-specific cell-mediated and antibody responses, including MHC class I restricted CTL. The results of this study are reported in this paper.

#### MATERIALS AND METHODS

**Production and purification of rHIV-1 envelope proteins and synthetic peptides.** rHIV-1<sub>env</sub> gp160 was expressed using the BH8 clone (20) as several truncated forms containing different deletions (Fig. 1). The truncated HIV-1 160D and 448C proteins were expressed using a baculovirus expression system in sf9 cells (21). The HIV-1 160D was produced routinely in a glycosylated form, but the nonglycosylated form was also produced by growing sf9 cells in the presence of 1  $\mu$ g/ml tunicamycin. The 448C protein was expressed normally as a nonglycosylated protein. The BD, AD, AD44, and ADBH proteins were expressed in *Escherichia coli* as variations of the CBre3 protein, the Ag in HIV-1 diagnostics (22).

Recombinant proteins were purified as denatured, reduced, and chemically modified proteins from sf9 cell lysates or from *E. coli* inclusion bodies using standard chromatography techniques (23). Recombinant proteins purified in this way were not soluble in aqueous buffers and were dissolved in 50 mM borate buffer, pH 8.0, containing 8 M urea. Lysates from noninfected sf9 cells were sham purified using the same procedure, and the protein that was recovered was used as the negative control Ag preparation for all immunoassays.

Peptides containing the immunodominant CTL epitope from the HIV-1<sub>env</sub> V-3 loop (24), 18IIIIB peptide (amino acid sequence RIQRGPGRFVITIGK), and the CTL epitope from OVA (25), OVA<sub>258-278</sub> (amino acid sequence INFELKLEWTSSNVMEER), were synthesized on a PSS-80 peptide synthesizer (Applied Protein Technologies, Cambridge, MA) using standard *t*-butoxycarbonyl chemistry (26). The synthetic peptides were purified by HPLC and characterized by amino acid analysis.

**Purification of saponin fractions.** Crude *Quillaja* powdered extract (Penco, Lyndhurst, NJ) was used as the starting material. The total saponin fraction was purified by membrane ultrafiltration to remove low m.w. contaminants. Individual saponins were purified further by adsorption chromatography through silica and reverse phase HPLC (19). These fractions contained only a single major saponin, based on reverse phase HPLC criteria. A single fraction known to contain an adjuvant active saponin, QS-21, was selected for this study.

**Formulation of experimental vaccines.** The experimental vaccine formulations were based on alum-adsorbed HIV-1 160D. The purified glycosylated HIV-1 160D protein, in 50 mM borate buffer containing 8 M urea, was diluted using 0.1 M sodium bicarbonate buffer, pH 8.8, in the presence of the appropriate quantity of 2% alum, Al(OH)<sub>3</sub> (Alhydrogel, Superfos, Accurate Chemical and Scientific Corp., Westbury, NY), to reduce the urea concentration to 2 M and incubated at room temperature for 24 h. The protein-coated alum was pelleted by centrifugation at 400  $\times$  g, for 10 min, washed, and

resuspended in sterile saline. The amount of protein bound to precipitated alum was determined directly (BCA assay, Pierce Chemical Co., Rockland IL). Under these conditions the binding of protein to alum was very efficient; routinely > 95% bound. A protein:alum ratio of 1:10 was used for our studies.

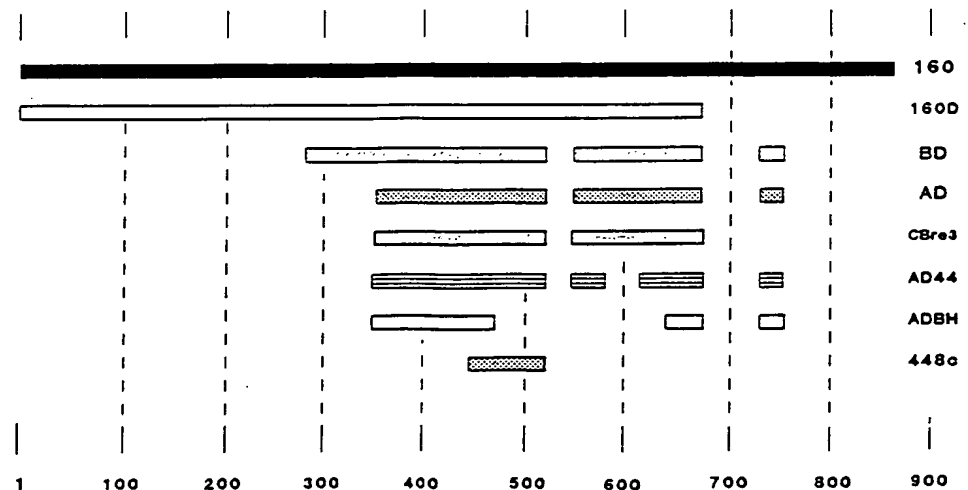
**Experimental vaccination protocol.** Female BALB/c mice, 8 to 10 wk of age, were immunized in groups of 10 for each experiment, and all experiments were repeated using strain-, age-, and sex-matched groups. Each mouse dose of the HIV-1 experimental vaccine contained 25  $\mu$ g of HIV-1 160D adsorbed to 250  $\mu$ g of alum and was mixed with 10  $\mu$ g of QS-21, when this was used. Mice were immunized s.c. two times at 2-wk intervals with the different vaccine formulations. Adjuvant control immunizations, consisting of 10  $\mu$ g of QS-21 in saline, were given using the same schedule. Separate groups of vaccinated mice were sham challenged by immunizing mice s.c. with 50  $\mu$ g of heat-inactivated (56°C for 60 min), gradient-purified HIV-1<sub>env</sub> (Advanced Biotechnologies, Columbia, MD) 8 wk after the second vaccination.

Serum was recovered 2 wk after the second immunization or 1 wk after sham challenge by cardiac puncture from anesthetized mice and used as the source of antibodies. Spleens were removed aseptically and dispersed into mononuclear cell suspensions. Mononuclear cells were washed and resuspended at a concentration of  $2 \times 10^6$ /ml in culture media consisting of RPMI 1640 (GIBCO, Grand Island, NY) buffered with NaHCO<sub>3</sub> and supplemented with 10% (v/v) FCS (HyClone Laboratories, Logan, UT), 2 mM L-glutamine (GIBCO), 20  $\mu$ M 2-ME (Sigma), and 50  $\mu$ g/ml gentamicin sulfate (Schering Corp., Kenilworth, NJ).

**Measurement of HIV envelope-specific antibodies.** Murine antibody responses were measured by ELISA using both glycosylated and nonglycosylated HIV-1 160D and the truncated and deleted proteins expressed in *E. coli* as the test Ag and purified sf9 cell debris as the background control. Proteins were diluted in 0.1 M sodium bicarbonate buffer, pH 8.8, to a concentration of 4  $\mu$ g/ml. The proteins were passively adsorbed to the wells of Immulon-2 assay plates (Dynatech, Chantilly, VA) by incubation of 50  $\mu$ l/well for 18 h at room temperature. Sera from individual mice were diluted in PBS containing 4% (w/v) BSA using a log<sub>2</sub> titration scheme, serum dilutions of 1/25 to 1/390,625 (5<sup>2</sup> to 5<sup>9</sup>) were routinely tested. Antibody binding to test Ag was detected using horseradish peroxidase avidin-biotin-conjugated reagents (Vectastain ABC, Vector Laboratories, Burlingame, CA) (27). Serum samples from individual mice were tested in quadruplicate at all dilutions and used to determine the mean and SD of absorbance values. Absorbance values were considered as significant when the mean value for each serum dilution was greater than the mean absorbance values obtained using the same serum on the sf9 cell control protein. Titer end points were established as the highest dilution of serum used in which significant levels of antibody binding were measured.

**Measurement of Ag-specific cellular proliferation.** Assays were done using round bottom 96-well microculture plates (Costar, Cambridge, MA) and splenic mononuclear cells at a concentration of  $1 \times 10^6$ /ml in total culture volumes of 200  $\mu$ l, which included the appropriate concentrations of test or control Ag. Cultures were maintained at 37°C in a humid incubator with 5% CO<sub>2</sub> in air, and the total

**Figure 1.** Recombinant proteins used for these studies included 160D, BD, AD, CBre3, AD44, ADBH, and 448C; full length HIV-1 gp160 is shown for reference. The 160D protein contained HIV-1 envelope amino acids 1 to 674 (BH8 clone) and was produced in sf9 cells. The BD, AD, CBre3, AD44, and ADBH proteins were expressed in *E. coli*. The hydrophobic region containing amino acids 524 to 545 was deleted in all of these *E. coli*-expressed proteins to increase protein production levels. The BD protein contained amino acids 281 to 523, which includes the V-3 loop: 546 to 674, which includes the gp41 immunodominant region, and 730 to 752. The AD protein was similar but without the V-3 loop region; it started at amino acid 350. The CBre3 protein was similar to the AD protein, but the region consisting of amino acids 730 to 752 was not present. The AD44 and ADBH proteins were also similar to the AD protein but were deleted further to remove the gp41 immunodominant region, amino acids 586 to 615 and 473 to 647, respectively. The 448C protein contained only amino acids 448 to 518 and was expressed in sf9 cells as a tetramer.



culture time was 144 h. One  $\mu$ Ci of [ $^3$ H]thymidine (ICN Radiochemicals, Irvine, CA) was added to all wells 16 h prior to harvesting the cultures, and cellular proliferation was quantitated by standard liquid scintillation counting. Mice were tested individually, and all culture permutations were run in quadruplicate.

Test Ag used for proliferation assays included rHIV-1 proteins and inactivated HIV-1 virus preparations. The denatured rHIV-1 proteins were not soluble in aqueous buffers so they were adsorbed to 1.0- $\mu$ m Polybead-hydroxylate latex microspheres (Polysciences Inc., Warrington, PA) (37). Conditions were controlled so that  $10^6$  latex microspheres were coated with 1  $\mu$ g of protein. The Ag-coated particles were diluted in complete culture media to the desired assay concentrations and were used *in vitro* at different particle:mononuclear cell ratios to provide a titration of Ag.

Three variants of HIV-1 (IIIB, MN, and CC) were produced in cell culture using infected H9 cells (AIDS Research and Reference Reagent Program, Rockville, MD). HIV-1 Ag used for proliferation assays consisted of heat-inactivated (56° C for 60 min) supernatant fluids from cultures of infected H9 cells, and supernatant fluid from cultures of noninfected H9 cells was used as the negative control. The concentration of HIV-1 was adjusted to 1  $\mu$ g/ml using p24 gag as the standard (Coulter Immunology, Hialeah, FL); the actual amounts of gp120, gp41, or gp160 in the culture supernatant were not determined.

**Measurement of Ag-specific CTL.** Effector CTL were generated by culture of pooled splenic mononuclear cell from two to five mice with 3.0  $\mu$ M 18IIB peptide; the OVA<sub>258-276</sub> peptide was used similarly as the negative control. Culture was done using 2-ml volumes,  $10^6$  cells/ml, in 15-ml culture tubes (Corning Glass, Corning, NY). Cells were recovered after a 144-h culture, washed, resuspended in fresh media, and used in the CTL assay.

Murine P815 cells, which express only class I MHC Ag, were obtained (ATCC, Rockville, MD) and used for CTL targets. Cells were infected with either recombinant vaccinia virus (vPE8), which contains the HIV-1<sub>160D</sub> gp120 gene, or control vaccinia virus (AIDS Research and Reference Reagent Program) and used as the target cells. Infection was done by incubating the needed numbers of target cells with 5 to 10 PFU/cell of vaccinia virus at 37° C for 1 h at a cell concentration of  $10^7$ /ml. Infected cells were diluted to  $0.5 \times 10^6$ /ml and cultured 18 h prior to  $^{51}$ Cr labeling.

Vaccinia-infected cells were labeled ( $10^6$  cells/experiment) with 250  $\mu$ Ci of Na<sub>2</sub>CrO<sub>4</sub> ( $^{51}$ Cr, 534 mCi/mg, Du Pont Co., Wilmington, DE) by incubation for 1 h at 37° C in culture media containing 0.3 M sucrose in a volume of 0.5 ml. A standard 4-h cytotoxicity assay format was used with  $10^4$  target cells/well and a titration of E:T ratios ranging from 25:1 to 1.5:1 (28), and the percent specific release was determined (24). The CD8<sup>+</sup> phenotype of CTL was demonstrated by selectively killing mature CTL using mAb to either CD4 or CD8 (Becton-Dickinson, San Jose, CA) plus guinea pig C (Accurate Chemical and Scientific Corp.).

**Measurement of CTL maturation *in vitro*.** The increased production of serine esterase which associated with Ag-specific CTL maturation and activation (29, 30) was measured as a secondary assessment of CTL function. Murine splenic mononuclear cells were cultured using the same protocol described for measuring Ag-specific proliferation. After a total culture of 168 h, cellular contents of each of the wells were washed twice with PBS, resuspended in 100  $\mu$ l of 0.2 M Tris-HCl, pH 8.8, and stored at -20° C. Serine esterase activity present in the cell lysates was measured using a colorimetric assay (29). Background values were determined using splenic mononuclear cells cultured with either sf9 cell protein or control supernatant fluid from H9 cell cultures as the Ag, and the results were subtracted from those obtained using recombinant or viral proteins.

**Statistical analysis.** Variation of Ag-induced proliferative and serine esterase responses was distributed normally within test groups. The significance of apparent differences between test groups was established using Student's *t*-test. Analysis of antibody titers and percent specific release data from CTL assays was done using the Mann-Whitney U test because the data were not distributed normally. All analyses were done using the ABstat computerized statistical program (AndersonBell, Parker, CO).

## RESULTS

**Measurement of total specific antibody titers.** Antibody responses produced after immunization with the experimental vaccine formulations were measured using ELISA and recombinant proteins (Fig. 2, left). The mice vaccinated twice with the formulation containing QS-21 consistently produced antibodies to titers that were 25- to 125-fold higher than those vaccinated with alum-adsorbed HIV-1 160D; this difference was marginally sig-

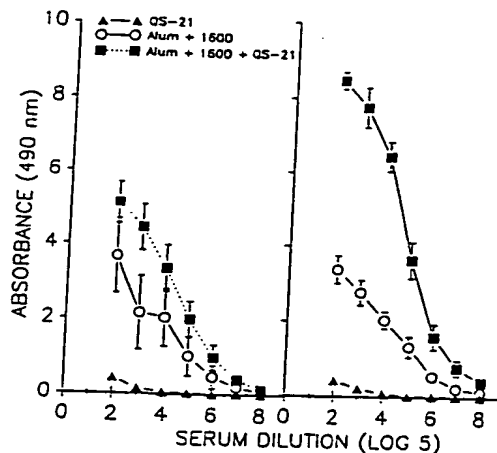


Figure 2. Measurement of mouse serum antibodies specific to HIV-1 160D by ELISA after vaccination with either alum-adsorbed HIV-1 160D (○—○), the complete vaccine formulation containing alum-adsorbed HIV-1 160D + QS-21 (■—■), or QS-21 alone (Δ—Δ). Serum samples from individual mice were assayed separately; the data shown represent the mean  $\pm$  1 SD of absorbance (490 nm) readings of mice from each group. Dilutions of serum are shown as log<sub>5</sub> dilutions. Responses produced after vaccination are shown in the left panel; amnestic responses produced after sham challenge with inactivated HIV-1<sub>160D</sub> are shown in the right panel.

nificant ( $p \leq 0.05$ ). Antibody titers were increased significantly ( $p \leq 0.01$ ) 1 wk after sham challenge with inactivated HIV-1<sub>160D</sub> but only for mice that had been vaccinated with the QS-21 containing formulation (Fig. 2, right). The increases in total antibody titers were 625- to 3125-fold when comparisons were made between individual mice in the test groups.

**Specificity of antibody responses.** Antibody binding to different regions of HIV-1 gp160 was measured by ELISA using both recombinant proteins and synthetic peptides as the test Ag. Serum samples from the mice that had received the vaccine containing QS-21 had higher levels of specific antibodies to all of the recombinant proteins when compared with mice that received alum-adsorbed HIV-1 160D (Fig. 3). This pattern did not change after sham challenge with inactivated HIV-1<sub>160D</sub> (data not shown).

**Ag-specific proliferation.** Splenic lymphocytes from immunized mice proliferated in response to both glycosylated and nonglycosylated HIV-1 160D (Fig. 4). Significant proliferative responses were observed at high Ag concentrations for the mice that had been immunized with only the alum-adsorbed HIV-1 160D. However, responses of mice immunized with the complete vaccine formulation were significantly increased ( $p \leq 0.02$ ) when lower concentrations of Ag were used. Significant proliferation to control sf9 cell protein was not observed, nor did mononuclear cells from naive and adjuvant control mice proliferate (data not shown). Proliferative responses to HIV-1 viral proteins were also produced but only if the complete vaccine formulation was used to immunize mice; the difference between groups was highly significant ( $p \leq 0.01$ ). Proliferative responses appeared to be group specific because they were induced *in vitro* using three different variants of HIV-1.

**CTL responses.** Splenic mononuclear cells from immune mice were capable of killing P815 cells infected with the recombinant vaccinia virus containing the HIV-1 gp120 gene. Specific CTL could not be detected in fresh

Figure 3. Antibody responses to truncated and deleted recombinant proteins after two immunizations. Serum samples from individual mice were assayed separately; data shown represent the mean  $\pm$  1 SD of  $A_{490\text{ nm}}$  readings from ELISA using a single serum dilution of 1/25 and individual serum samples from all of the mice in each of the test groups. Groups are shown based on the vaccine formulation used. The open bars represent data from mice immunized with alum-adsorbed HIV-1 160D, and the hatched bars represent data from mice immunized with the same vaccine formulation supplemented with QS-21.

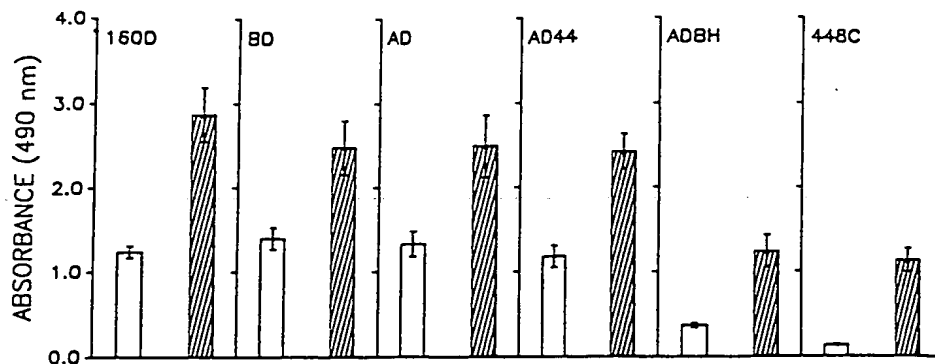
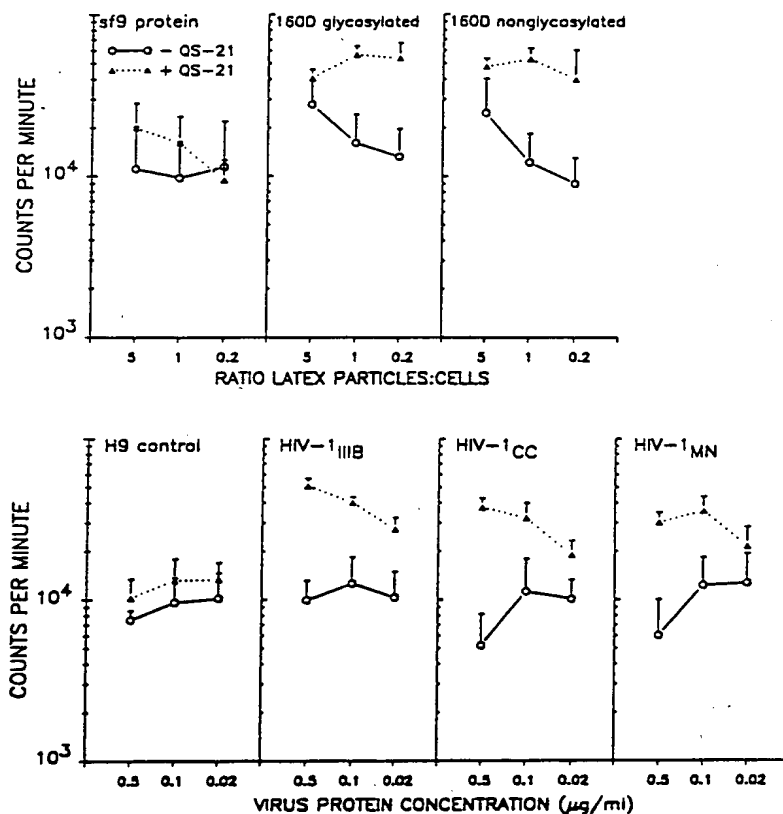


Figure 4. Proliferative responses of murine splenic mononuclear cells to sf9 control protein, glycosylated, non-glycosylated HIV-1 160D, and inactivated HIV-1 virus preparations. Responses of mice that received the complete vaccine are shown as ( $\Delta$ — $\Delta$ ); those that received only the alum-adsorbed HIV-1 160D are shown as ( $\circ$ — $\circ$ ). Ag quantity is shown as the ratios of Ag-coated particles:cells for recombinant proteins (top panel) and as protein concentrations,  $\mu\text{g}/\text{ml}$  final concentration in vitro, for inactivated HIV-1 viruses (bottom panel). Proliferation is shown as cpm ( $\log_{10}$ ). All mice in the test group were tested individually using quadruplicate cultures and a titration of Ag. The data represent the mean  $\pm$  1 SE of total cpm for all mice in individual test groups and are shown according to the Ag used in vitro to induce responses.



spleen cells but were induced to differentiate into mature CTL by stimulation in vitro with the 18IIIIB peptide (Fig. 5); the OVA peptide did not induce measurable CTL responses (data not shown). The CTL responses were specific to HIV-1 gp120 inasmuch as target cells infected with control vaccinia virus were not killed. CTL responses were not generated in mice immunized with only alum-adsorbed HIV-1 160D. mAb specific for CD8 reduced CTL activity to background levels whereas the CD4-specific mAb or C alone did not affect CTL function (data not shown).

**Production of serine esterase as a measure of CTL maturation.** Increased production of serine esterase was detected after splenic mononuclear cells from immune mice were cultured with either glycosylated or nonglycosylated HIV-1 160D (Fig. 6). Significantly higher levels were produced using mice immunized with the complete vaccine ( $p \leq 0.02$ ). Serine esterase produced in response

to HIV-1 viral proteins was detected only for mice that had received the complete vaccine. These responses were similar to the proliferative responses in that they were raised against different HIV-1 variants, suggesting that they were group specific.

**Specificity of cell-mediated immune responses.** In vitro proliferative responses to the truncated and deleted proteins were variable, but the use of QS-21 in the vaccine formulation significantly increased responses to all of the proteins, including ADBH, which did not induce proliferation of splenic mononuclear cells from mice immunized with only alum-adsorbed HIV-1 160D (Fig. 7). These data suggest that the use of QS-21 in the vaccine formulations induced recognition of at least one epitope that was not immunogenic when presented with alum alone.

The production of serine esterase by mononuclear cells from mice that received the complete vaccine could be

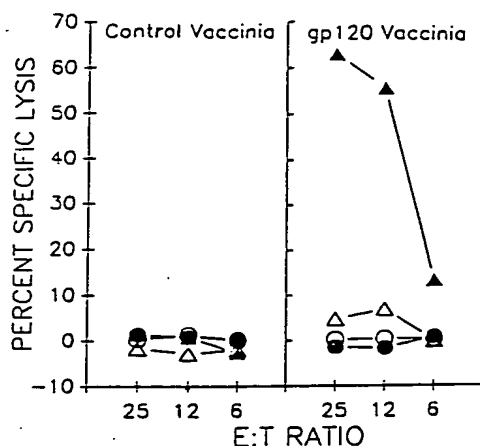


Figure 5. CTL function was measured using P815 cells infected with control vaccinia viruses (left panel) or recombinant (vPE8) vaccinia virus (right panel) as the target cells. Responses using mice immunized with only the alum-adsorbed HIV-1 160D are shown as (○—○, ●—●); those receiving the complete vaccine are shown as (Δ—Δ, ▲—▲). Effector cells were splenic mononuclear cells used after a 144-h culture, with the 18IIIIB peptide (●—●, ▲—▲) or with the OVA<sub>238-378</sub> peptide (○—○, Δ—Δ). Data are shown as percent specific release using a titration of E:T ratios.

induced in vitro by several of the recombinant proteins, even those with extensive deletions (Fig. 7). The pattern of these responses suggested that one of the immunodominant regions in either the V-3 loop or the gp41 immunodominant region was needed to induce CTL maturation, based on the lack of responses to the AD44 and ADBH proteins. However, responses to the 448C tetrameric protein were induced in the group that received the complete vaccine formulation, suggesting that epitopes, possibly minor epitopes, recognized by CTL are present in this region. We found that significant CTL maturation was not induced in vitro by any of the deleted proteins using splenic mononuclear cells from mice immunized with the alum-adsorbed HIV-1 160D. The QS-21 adjuvant appeared to induce recognition of epitopes that were not immunogenic when adsorbed to alum, similar to the results observed for proliferative responses but more pronounced using the production of serine esterase as the measure.

#### DISCUSSION

The results of this study demonstrated that the QS-21 saponin fraction contains a potent adjuvant that functioned in the presence of alum and significantly increased Ag-specific immune responses to HIV-1 160D. Experimental vaccine formulations containing QS-21 adjuvant augmented antibody responses and primed the murine immune system to respond to HIV-1 viral proteins in an amnestic fashion (Fig. 2), characteristics critical for efficacy of viral vaccines. Specificity analysis of serum antibodies, done using truncated and deleted recombinant proteins, demonstrated that the inclusion of QS-21 into the vaccine formulation increased the production of antibodies to many different regions on HIV-1 gp160, but it did not significantly increase the recognition of different epitopes (Fig. 3).

The addition of the QS-21 adjuvant to the vaccine formulation significantly augmented proliferative responses to both glycosylated and nonglycosylated HIV-1 160D (Fig. 4). The most significant difference was that

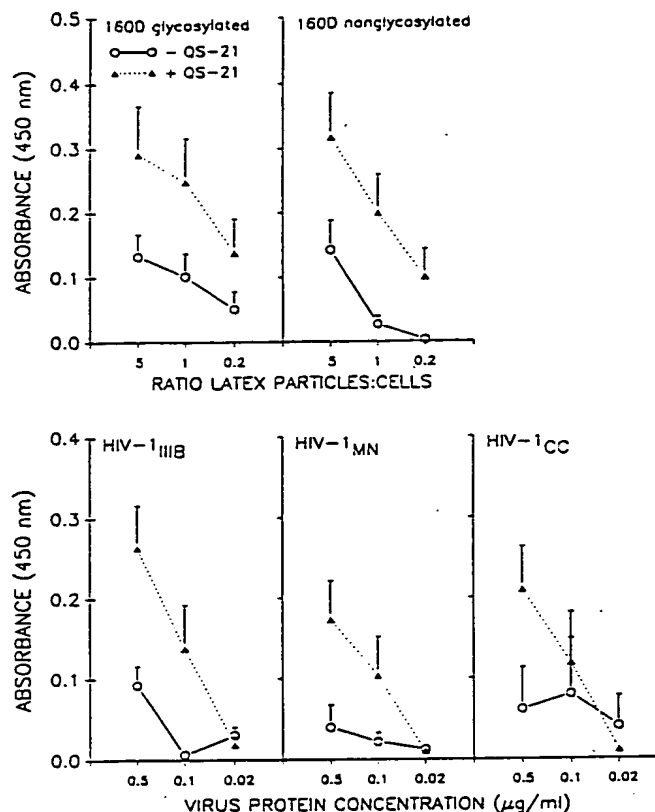


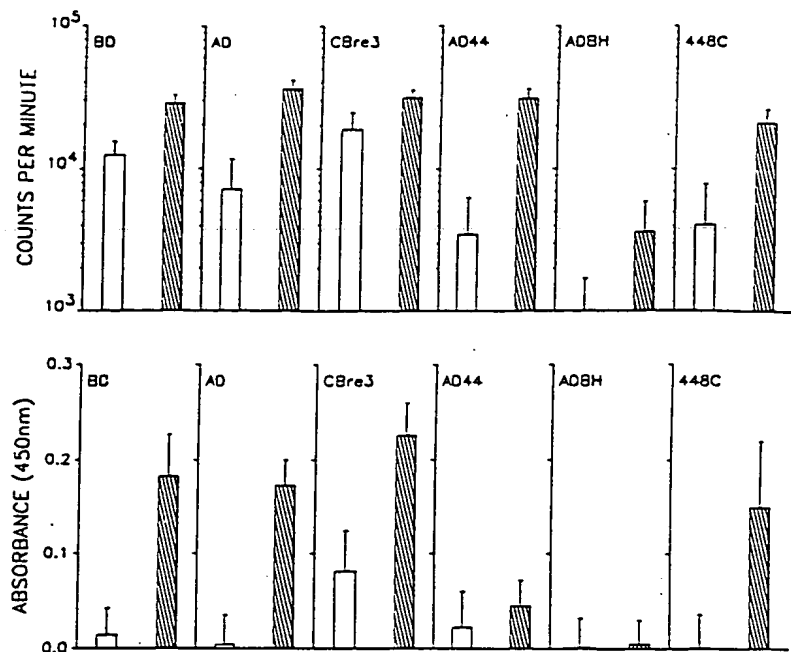
Figure 6. Serine esterase produced in vitro in response to glycosylated and nonglycosylated recombinant HIV-1 160D adsorbed to latex particles or to inactivated HIV-1 virus preparations. All mice in the test group were tested individually using quadruplicate cultures and a titration of Ag, shown as the ratios of Ag-coated particles: cells for recombinant proteins (top panel) and as viral protein concentrations,  $\mu\text{g/ml}$  final concentrations in vitro, for inactivated HIV-1 viruses (bottom panel). Responses of mice that received the complete vaccine are shown as (▲—▲); those that received only the alum-adsorbed HIV-1 160D are shown as (○—○). The data represent the net  $\pm$  1 SE of  $A_{450\text{ nm}}$  readings from all mice in individual test groups and are shown according to the protein used in vitro.

proliferation to much lower concentrations of Ag was induced when the mice were immunized with the complete vaccine formulation. This finding suggests that the QS-21 induced the production of higher numbers of Ag-primed lymphocytes in vivo, particularly Th cells, or that the responding cells had higher affinity Ag receptors. These same mechanisms could also account for the increases in antibody responses, in which the induction of Th cells has been shown to augment HIV-1 peptide-specific antibody responses (31). However, processing and presentation of Ag could also have been augmented in vivo by the QS-21 adjuvant. Experiments using limiting dilution analysis are currently being done to address this point.

Significant group-specific proliferative responses to HIV-1 viral proteins were observed only using mice immunized with the formulation containing QS-21 (Fig. 4). These results document the ability of denatured recombinant proteins to induce cell-mediated immune responses to authentic HIV-1 proteins when used with the appropriate adjuvant, in this case a purified saponin. The group-specific aspect of the response could be very important because genetic variability of HIV-1 is considered to be a major obstacle to the development of an HIV-1



Figure 7. Proliferative and serine esterase responses to the *E. coli*-expressed recombinant proteins were measured using recombinant proteins, adsorbed to 1.0- $\mu$ m latex particles, as the Ag source. The proliferation data (top panel) represent the mean  $\pm$  1 SE of total cpm, and the serine esterase data (bottom panel) represent the net - mean  $\pm$  1 SE of  $A_{450\text{nm}}$  readings for all mice in individual test groups using the Ag-coated particle:cell ratio of 5:1, which was found to be optimal for inducing both proliferation and the production of serine esterase. Data are shown grouped according to the protein used in vitro. Responses of mice receiving the complete vaccine are shown as the hatched bars; those receiving only the alum-adsorbed HIV-1 160D are shown as the open bars.



vaccine (32).

HIV-1 type-specific CTL responses were produced in mice after vaccination with the alum-adsorbed HIV-1 160D mixed with QS-21 adjuvant and were readily detected using P815 cells infected with the vPE8 recombinant vaccinia virus as the targets (Fig. 5). These CTL responses were MHC class-I Ag restricted because the P815 cells do not express class-II Ag. The mechanisms through which the QS-21 adjuvant induced class-I MHC Ag-restricted CTL are not known. We believe that the QS-21 adjuvant may have increased or altered the activity of APC, particularly a subpopulation that is capable of presenting soluble protein Ag to class I MHC Ag-restricted CTL (33). Similar CTL responses have been induced using ISCOM (18) which are flattened ring-shaped micellar particles consisting of cholesterol, phospholipid, and saponins. It had been assumed that the adjuvant activity of ISCOM is primarily a function of their shape (17). However, the results of our studies indicate that the saponin component may be sufficient to generate class I MHC Ag-restricted CTL.

The use of QS-21 in the vaccine formulation significantly increased Ag-induced maturation of precursor CTL, which was measured as an increase in serine esterase production. These responses were similar to the proliferative responses inasmuch as only mice that had received the complete vaccine produced responses against HIV-1 viral proteins, and these responses were group specific (Fig. 7). Our studies indicate that the production of serine esterase was Ag specific. However, this type of assay does not allow for the identification of the responding cells, which may have been either CD4- or CD8-positive CTL. The induction of CD4-positive MHC class II Ag-restricted CTL in vaccinated humans has been demonstrated using cloned cell lines (14), and we assume that CTL with this same phenotype were produced by the immunized mice.

Recombinant proteins with deletions were used to determine the relative importance of individual regions

within the HIV-1 gp160 for inducing proliferative responses and the production of serine esterase. Our initial hypothesis was that the use of QS-21 would broaden the specificity of the immune responses so that both immunodominant and minor epitopes would be recognized. We found that recognition of all of the deleted recombinant proteins increased, in terms of total proliferative responses (Fig. 7), and the pattern of responsiveness was not strictly correlated with the presence or absence of the immunodominant V-3 region or other defined epitopes. The specificity appeared to be broadened only slightly, based on responses to a single extensively deleted protein, ADBH, that was immunogenic in mice receiving the complete formulation but not in mice immunized with only the alum-adsorbed HIV-1 160D. However, the QS-21 adjuvant appeared to increase significantly the recognition of additional epitopes when the production of serine esterase by Ag-stimulated mononuclear cells was measured. Based on these data, we believe that the QS-21 adjuvant did increase the recognition of weakly immunogenic epitopes, especially those recognized by cells with cytolytic capabilities.

Saponins from *Q. saponaria* have been identified as potent adjuvants and have been studied most thoroughly in the form of Quil A. This saponin mixture has been shown to augment antibody responses to both T-dependent and T-independent Ag and to induce Ag-specific helper T lymphocyte memory (34, 35). Our data were similar and suggest that we have identified and purified a major adjuvant-active fraction in *Q. saponaria* saponins. The potential usefulness of saponins as an adjuvant for HIV-1 vaccines has been strengthened recently by the results of studies using Quil A as a component in a simian immunodeficiency virus vaccine (36). The use of individual purified saponin fractions is technically simple, and the ability to manufacture a uniform adjuvant product is feasible. A similar experimental vaccine trial is now under way to characterize the adjuvant activity and safety of QS-21 in nonhuman primates.

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